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RESEARCH ARTICLE

Combination of a new generation of PNAs with a peptide-based carrier enables efficient targeting of cell cycle progression

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The design of potent systems for the delivery of charged and noncharged molecules that target genes of interest remains a challenge. We describe a novel technology that combines a new generation of peptide nucleic acids (PNAs), or HypNA-pPNAs, with a new noncovalent peptide-based delivery system, Pep-2, which promotes efficient delivery of PNAs into several cell lines. We have validated the potential of this technology by showing that Pep2-mediated delivery of an antisense HypNA-pPNA chimera directed specifically against cyclin B1 induces rapid and robust downregulation of its protein levels and efficiently blocks cell cycle progression of several cell lines, as well as proliferation of cells derived from a breast cancer. Pep-2-based delivery system was shown to be 100-fold more efficient in delivering HypNA-pPNAs than classical cationic lipid-based methods. Whereas Pep-2 is essential for improving the bioavailability of PNAs and HypNA-pPNAs, the latter contribute significantly to the efficiency and specificity of the biological response. We have found that Pep-2/HypNA-pPNA strategy promotes potent antisense effects, which are approximately 25-fold greater than with classical antisense oligonucleotide directed specifically against the same cyclin B1 target. Taken together, these data demonstrate that peptide-mediated delivery of HypNA-pPNAs constitutes a very promising technology for therapeutic applications.

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Keywords: *peptide-based delivery system; PNA; cyclin B1; cell cycle regulation; antisense; cancer therapy*

Introduction

Antisense technology holds a strong promise for therapeutic applications. Nevertheless, the efficiency of antisense oligonucleotides remains limited by their rapid degradation by intracellular nucleases, insufficient target affinity, nonspecific side effects and by their inefficient uptake due to the low permeability of the cell membrane.[1–3] In order to overcome these limitations, novel oligonucleotide chemistries have been developed and several new generations of antisense oligonucleotide have been proposed;[4,5] in particular, peptide nucleic acids (PNAs) that constitute very promising tools for antisense therapy in both eukaryotic and prokaryotic cells.[6,7] In PNAs, the phosphodiester backbone of DNA or RNA is replaced by a hybrid molecule corresponding to *N*-(2-aminoethyl) glycine monomers linked by amide bonds.[8,9] PNAs allow specific gene targeting, are highly stable, resistant to nucleases and proteases, and bind RNA and DNA targets in a sequence-specific manner with high affinity. However, only a few reports actually associate a strong biological response with the use of PNAs *in vivo*. The inefficiency of PNAs *in vivo* is in part related to their poor propensity to cross the cell membrane and/or to their inappropriate cellular localization.[10] Recent work has demonstrated that cellular uptake of free PNAs can occur, although this requires very high concentrations of PNAs, except in neuronal cells,[11,12] and yields limited biological response.[13–15] In order to improve delivery of PNAs into cells and to reduce the doses required for detectable effects, several chemical modifications based on covalently linked cell-penetrating peptides have been successfully used.[10,12,16–18] Linkage of nuclear localization or polylysine sequences to PNAs have also been reported to improve both their cellular internalization and nuclear translocation.[14,19,20] Moreover, it has recently been shown that the cellular uptake of PNAs depends on the cell type and that a carrier system is required for efficient uptake.[18] The solubility of PNAs and their tendency to self-aggregate are important factors to be considered for the biological use of PNAs.[21–23] PNA solubility is often sequence-dependent, which usually leads to several restrictions in PNA probe design and can be improved by incorporation of Lys residues or ethylene glycol linkers (eg1 = 8 amino-2-6 dioxaoctanoic acid).[18,21–24] Efimov *et al*[25,26] have developed a novel oligonucleotide mimic, a dimeric oligomer that consists of a phosphonate analog of PNA (*p*PNA) and a PNA-like monomer based on a *trans*-4-hydroxyl-L-proline (HypNA). The introduction of negative charges into the PNA backbone yields excellent solubility without hindering its other properties. This HypNA-*p*PNA chimera interacts with

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target sequences in a manner same as classical PNAs, independently of the ionic strength, and exhibits high thermal stability when complexed with DNA or RNA targets.[25–27]

The design of efficient systems for the delivery of hydrophilic molecules into living cells remains an essential challenge. To this aim, we have previously described a peptide-based carrier system for protein transduction termed Pep-1.[28] Based on Pep-1, we have designed a new peptide carrier (Pep-2) that enables efficient delivery of both classical and charged PNAs into living cells. Here, we present a new technology that combines this noncovalent peptide-based delivery system with the new generation of PNAs described above, or HypNA-*p*PNAs. In order to demonstrate the potential of this technology, we have chosen to target a protein that is essential for cell cycle progression, cyclin B1. Pep-2-mediated delivery of an antisense HypNA-*p*PNA chimera directed specifically against cyclin B1 leads to rapid and robust downregulation of cyclin B1 at both the mRNA and protein levels and efficiently blocks cell cycle progression of several cell lines, including cells derived from a breast cancer. Taken together, our data demonstrate the specificity and efficiency of this novel technology, thereby emphasizing its potential for therapeutic applications.

Results and discussion

Design and delivery of PNA and HypNA-p*PNA* directed against cyclin B1

Cell cycle progression is driven by sequential activation of essential heterodimeric protein kinase complexes (Cdk/cyclin complexes).[29] Most of the drugs currently designed to target cell cycle progression are directed against the kinase activity of the Cdk subunits.[30] However, as such drugs generally tend to affect other cellular kinases nonspecifically, we have recently proposed to target the regulatory cyclin subunit instead, as a more appropriate means of improving both the selectivity and the efficiency of cell cycle inhibitors.[31] Cyclin B1, in particular, constitutes a key target for cancer therapy, as a component of the essential 'Mitosis Promoting Factor' together with protein kinase Cdk1.[31–33] In the present work, we therefore decided to design an 18-mer antisense PNA directed against cyclin B1, and which specifically targets the first codons of the open reading frame of the cyclin B1 gene (CAT CGG GCT TGG AGG GAT) so as to increase the probability that it would interfere with translation of the messenger RNA. A scrambled sequence (TAG GGA GGT TCG GGC TAC) as well as a sequence containing two mismatches (CAT CAG GCT TAG AGG GAT) were used as controls. HypNA-*p*PNA chimeras were used in this study (Figure 1a) and their efficiency towards cyclin B1 was compared to that of PNAs and classical antisense oligonucleotide.

Although PNAs may enter cells under specific conditions, the main limitation to their use in therapeutic applications remains as their low cellular uptake. To overcome this obstacle, we designed a new peptide carrier, called Pep-2 (K E T W F E T W F T E W S Q P K K K R K V), based on the Pep-1 system previously developed for the delivery of proteins into living cells.[28]

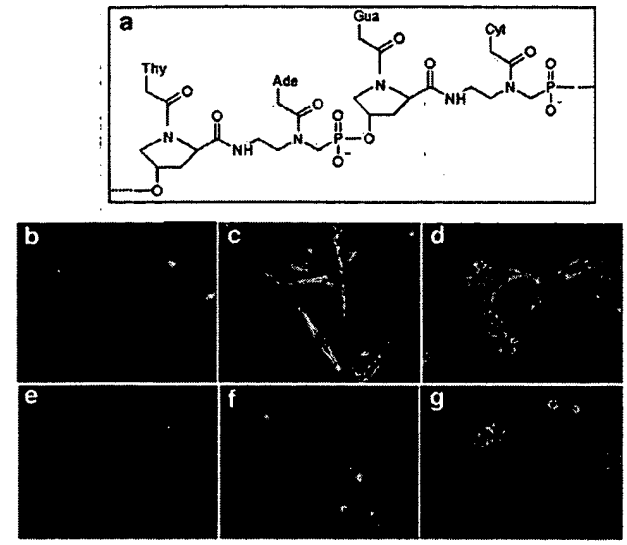

Figure 1 Pep-2-mediated delivery of HypNA-pPNA and PNA into mammalian cells. (a) Chemical structure of the PNA-related oligonucleotide mimic, HypNA-pPNA. A concentration of 0.5 μM of fluorescently labelled HypNA-pPNA (c, d) or PNA (f, g) were mixed with Pep-2 at a molar ratio of 1:25, incubated for 30 min at 37°C, then overlaid onto cultured cells for 1 h, after which cells were extensively washed prior to observation. Experiments were performed on both fixed (c, f) and living (d, g) cells. Control experiments illustrating inefficient uptake of free fluorescently labelled HypNA-pPNA (panel b) and PNA (panel e) (0.5 μM).

Pep-2 differs from Pep-1 by two Phe residues at positions 5 and 9, which replace Trp residues in the hydrophobic Trp-rich motif of Pep-1. We first evaluated the ability of Pep-2 to deliver PNAs and HypNA-*p*PNAs into cultured cells. An FITC-labelled HypNA-*p*PNA (0.5 μM) was incubated with a 25-fold molar excess of Pep-2 in serum-free medium for 30 min at 37°C. Cultured HS-68 or HeLa cells were then overlaid with the preformed complexes for 1 h in the presence of serum and examined by confocal microscopy. As fixation procedures have sometimes been reported to cause artifactual uptake,[18,34,35] experiments were performed on both fixed and living cells. In the absence of a carrier system, HypNA-*p*PNAs and PNAs barely entered cells (Figure 1b, e). In contrast, when associated with the carrier peptide Pep-2, both HypNA-*p*PNAs (Figure 1c, d) and PNAs (Figure 1f, g) were rapidly delivered into more than 90% of cells, in less than an hour and localized mainly to the cytoplasm with some staining in the nucleus. These data demonstrate that Pep-2 promotes efficient internalization of both HypNA-*p*PNAs and PNAs into cells, that this is not affected by the presence of serum, and that maximal uptake is achieved after 2 h. We evaluated the efficiency of different ratios of Pep-2 on the delivery of both HypNA-*p*PNAs and PNAs (Figure 2a). Maximal delivery efficiency was obtained for a molecular ratio between 20/1 and 30/1 depending on the cell line (Figure 2a). Accordingly, a molecular ratio of 25/1 was used in the rest of the work.

To investigate the mechanism through which Pep-2 mediates cellular uptake of PNAs or HypNA-*p*PNAs, we incubated cells with fluorescein-labelled PNAs or HypNA-*p*PNAs at either 37°C or at low temperature (5°C) for

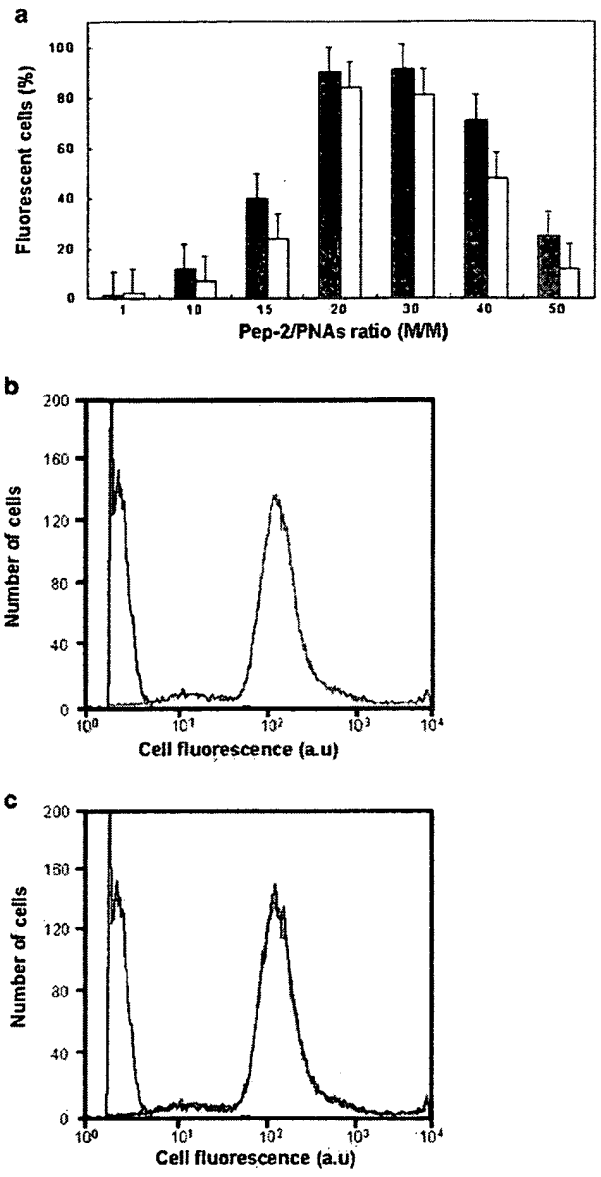

Figure 2 Mechanism of Pep-2-mediated cellular uptake of PNA and HypNA-pPNA. Concentration-dependent Pep-2 delivery of PNA and HypNA-pPNA. (a) A concentration of 0.1 μM of PNA (open bars) or HypNA-pPNA (closed bars) were incubated with increasing concentrations of Pep-2 from 0.1 μM (ratio 1:1) to 5 μM (ratio 50:1) as described in Figure 1. The efficiency of transfection was determined by counting fluorescent cells. (b and c) Mechanism of Pep-2-based HypNA-pPNA delivery. HeLa cells were incubated with 1 μM Pep-2/HypNA-PNA complexes for 30 min at either 37°C (panel b, green curve) or at low temperature (panel c, red curve). The blue curve corresponds to control cells in the absence of Pep-2/HypNA-pPNA. Cells were washed twice in PBS and treated with trypsin. Uptake of HypNA-pPNA was analysed by flow cytometry.

30 min and quantified the cellular concentration of fluorescently labelled HypNA-*p*PNAs by flow cytometry (Figure 2b, c). In order to avoid an overestimation of the cellular concentration of HypNA-*p*PNAs due to association of the complexes with the cell surface, cells were trypsinized and washed prior to analysis. No dramatic changes were observed between 5 and 37°C, and no reduction of the intracellular level of HypNA-*p*PNAs was observed at lower temperatures. These results suggest that in contrast to recent work published on Tat peptide,[36] the mechanism of cellular uptake of HypNA-*p*PNAs mediated by Pep-2 is independent of the endosomal pathway, at least for the first stage.

Antisense cyclin B1 HypNA-pPNA promotes robust downregulation of cyclin B1

We next examined whether Pep-2-mediated delivery of PNAs or HypNA-*p*PNAs targeting cyclin B1 yielded a robust biological response. To this aim, we first assessed whether cyclin B1 protein levels were downregulated by Pep-2-mediated delivery of HypNA-pPNAs, and to what extent, compared to delivery of PNAs and classical antisense oligonucleotide. The effect of increasing concentrations of antisense PNAs from 50 nM to 2 μM complexed with Pep-2 (ratio 1:25) or with classical cationic lipids on cyclin B1 protein levels was analysed in several cell lines, including HeLa cells, human fibroblasts (HS 68) and 293 cells. Pep-2/HypNA-*p*PNA and Pep-2/PNA complexes were overlaid onto asynchronous cultured cells in the presence of FBS (10%), and cyclin B1 protein levels were quantified by Western blotting 24 h after transduction. Cdk2 protein kinase was used as a control to normalize protein levels. Similar results were obtained for the three cell lines and typical Western blots for HS-68 fibroblasts are shown in Figure 3. Cyclin B1 protein levels were significantly reduced with 50 nM antisense HypNA-*p*PNA and completely abolished with 100 nM HypNA-*p*PNA complexed with Pep-2 (Figure 3a). In contrast, cyclin B1 protein levels were not affected by either 2 μM scrambled or mismatched HypNA-*p*PNA/Pep-2 complexes (Figure 3d, lane 2) or free HypNA-*p*PNA (Figure 3a, lane 2). Similar experiments performed with classical PNAs demonstrate that Pep-2 consistently improves the delivery and biological

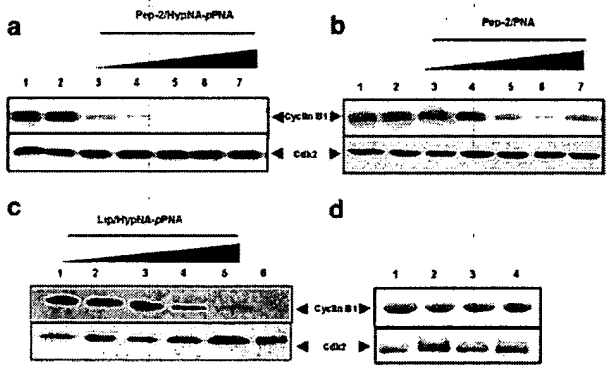

Figure 3 Pep-2-mediated delivery of PNA and HypNA-pPNA inhibits cyclin B1 expression. Increasing concentrations (lane 3: 50 nM, lane 4: 100 nM, lane 5: 500 nM, lane 6: 1 μM, lane 7: 2 μM) of HypNA-pPNA (a) or PNA (b) were incubated with Pep-2 at a molar ratio 1:25 at 37°C for 1 h, then overlaid onto cultured cells. Cyclin B1 protein levels were analysed by Western blotting after 24 h. Cdk2 protein was used as a control to normalize protein levels. Panel c: Lipofectamine was used for the delivery of different concentrations of HypNA-pPNA (lane 2: 200 nM, lane 3: 500 nM, lane 4: 1 μM, lane 5: 3 μM) and of 2 μM of PS-oligonucleotide (lane 6). Experiments performed with 2 μM free HypNA-pPNA (a, lane 2), PNA (b, lane 2). Panel d: Control experiments performed with 2 μM of scrambled HypNA-pPNA complexed to Pep-2 (lane 2), scrambled PNA (lane 3) complexed to Pep-2, and 2 μM of Pep-2 (lane 4). The level of endogenous cyclin B1 in untreated cells is reported in lane 1 of each panel.

efficiency of classical PNAs (Figure 3b). For comparison, cationic lipid-mediated delivery of HypNA-*p*PNA (Figure 3c) was much less efficient than Pep-2, as 500 nM of HypNA-*p*PNA was required to achieve significant reduction of cyclin B1 protein.

Quantification of cyclin B protein levels from these Western blots allowed us to estimate IC_{50} values for downregulation of cyclin B1, and thereby to compare the potency of Pep2/HypNA-*p*PNAs complexes with that of the other antisense strategies targeting cyclin B1 (Figure 4). As expected, Pep-2/HypNA-*p*PNA complexes have a potent antisense effect with an IC_{50} of 14 ± 5 nM, a value that is 8.5-fold and 25-fold lower than that of Pep-2/PNA (IC_{50}: 120 ± 24 nM) and classical phosphorothioate-oligonucleotide (IC_{50}: 360 ± 41 nM), respectively. Cationic lipid-mediated delivery of HypNA-*p*PNAs was 100-fold less efficient (IC_{50}: 1.3 ± 0.21 μM), consistent with data already reported by other groups using lipids[10,22,36] or covalently linked CPPs[5,10,18] for the delivery of standard PNAs. Taken together, these results demonstrate that Pep-2 constitutes a much more appropriate system for delivery of PNAs and HypNA-*p*PNA delivery. We have demonstrated that Pep-1 can also deliver PNAs into cells; however, the biological response is relatively poor in comparison to Pep-2, which suggests that the mutations of Pep-1 to Pep-2 are essential for either the interaction with the PNA or its rapid release within the cells, thereby enabling it to reach its target more efficiently (Figure 4).

In addition, we examined cyclin B1 levels and localization by indirect immunofluorescence. HS68 fibroblasts were synchronized by serum starvation, then restimulated to enter the cycle by the addition of fresh medium supplemented with serum and overlaid with HypNA-*p*PNA (0.5 μM) or preformed Pep-2/HypNA-*p*PNA (0.5 μM) complexes (molecular ratio 25/1) 4 h later. In both control cells and cells incubated with free HypNA-*p*PNA, after 24 and 48 h cyclin B1 was detected throughout the cells, but particularly strongly in the nucleus (Figure 5b, d, f and h). In contrast, cells treated

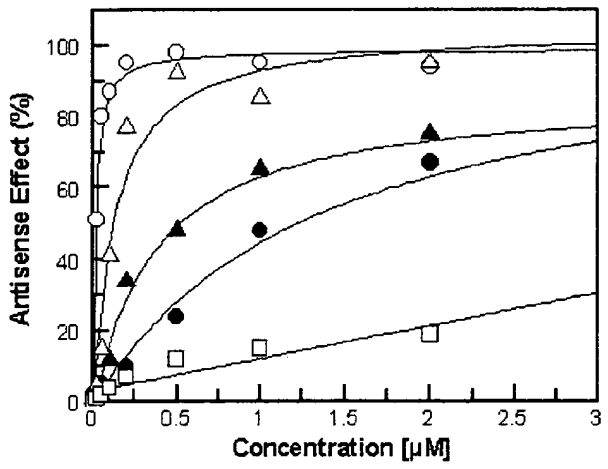

Figure 4 Potency of Pep2/HypNA-pPNA targeting cyclin B1. Comparative antisense effects of the different strategies aimed at targeting cyclin B were estimated using ImageQuant to quantify cyclin B levels acquired from Western blots in Figure 3 The values represent the average of three separate experiments. Pep-2/HypNA-pPNA (open circles), Pep-1/HypNA-pPNA (open boxes) Pep-2/PNA (open triangles), PS-oligonucleotide delivered with cationic lipids (closed triangles) and HypNA-pPNA delivered with cationic lipids (closed circles).

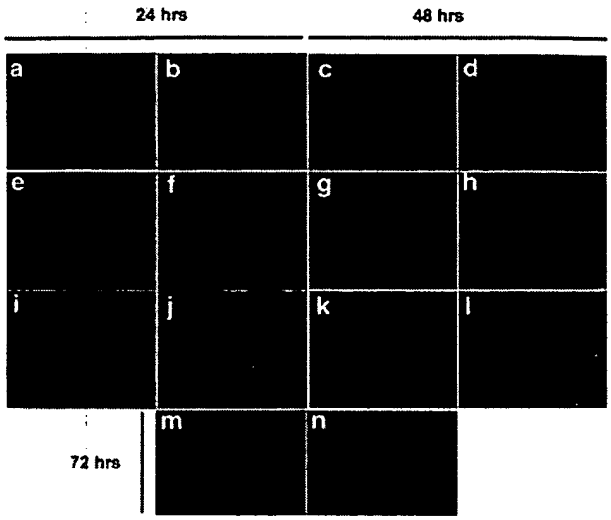

Figure 5 Pep-2-mediated delivery of HypNA-pPNA reduces intracellular levels of cyclin B1. HS-68 fibroblasts synchronized by serum starvation were restimulated to enter the cycle by addition of fresh medium supplemented with serum and overlaid with 0.5 μM HypPNA-pPNA alone or complexed to Pep-2 at a molecular ratio of 1:25 4 h later. Subcellular localization of cyclin B1 was examined by indirect immunofluorescence with anti-cyclin B1 antibody H-433 (Santa Cruz Biotechnology Inc.) 24, 48 and 72 h following transfection. Panels a–d show control, mock treated cells. Panels e–h show cells treated with 0.5 μM HypPNA-pPNA alone. Panels i–n are representative examples of cells treated with HypPNA-pPNA/Pep-2 complexes. Panels a, c, e, g, i, k and m: nuclear staining with Hoechst. Panels b, d, f, h, j, l and n: cyclin B1 staining.

with Pep-2/HypNA-*p*PNA, exhibited a significantly weaker nuclear staining of cyclin B1 after 24 h (Figure 5j), and complete disappearance of overall staining by 48 h (Figure 5l). Of particular relevance, downregulation of cyclin B1 protein was still apparent after 72 h (Figure 5n).

Antisense cyclin B1 HypNA-pPNA reduces cyclin B1 mRNA level

To provide further insight into the mechanism through which antisense HypNA-*p*PNA downregulates cyclin B1, we examined whether cyclin B1 mRNA was affected. Cyclin B1 mRNA levels were determined 15 h after incubation of the cells with either scrambled HypNA-pPNA/Pep-2 (2 μM) or HypNA-*p*PNA/Pep-2 (0.5 μM) complexes (Figure 6a). Upon incubation of HS68 fibroblasts with HypNA-*p*PNA/Pep-2, cyclin B1 mRNA levels decreased by more than 90%, whereas no changes were observed with mismatched HypNA-*p*PNA/Pep-2. Moreover, a time-course experiment revealed that the decrease in cyclin B1 mRNA levels was initiated after 5 h and that was reduced by more than 70% after 10 h (data not shown). These results reveal that the antisense HypNA-*p*PNA designed to target cyclin B1 not only induces a dramatic decrease in protein levels, but also affects cyclin B1 mRNA. In contrast to antisense oligonucleotides, PNAs have not been shown to trigger endogenous RNase-H activity; therefore, in order to investigate the mechanism of HypNA-*p*PNA action, we examined the ability of HypNA-*p*PNA to activate RNase-H and stimulate cleavage of cyclin B1 mRNA in an *in vitro* assay (Figure 6b). As a control, the phosphorothioate oligonucleotide induced cleavage of 70% of

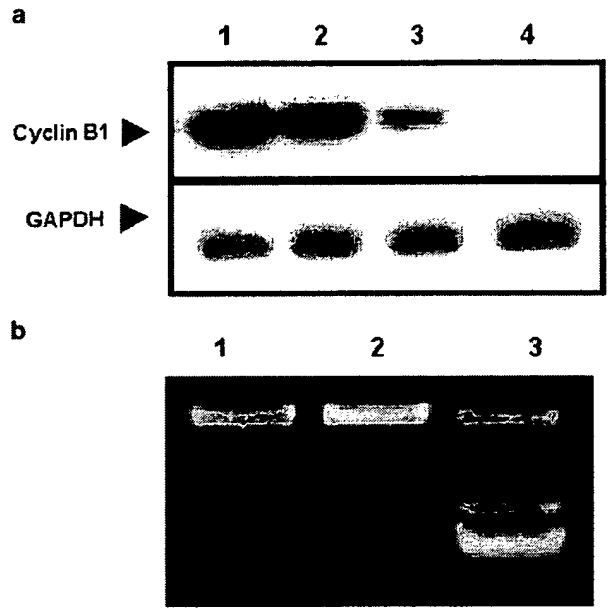

Figure 6 Pep-2-mediated delivery of HypNA-pPNA downregulates cyclin B1 mRNA. (a)Experiments were performed as described in Figure 2 and cyclin B1 mRNA levels were analysed by Northern blotting in the absence of antisense molecules (lane 1), in the presence of 2 μM mismatched HypNA-pPNA/Pep-2 (lane 2), and of 0.05 μM (lane 3) or 0.1 μM (lane 4) of HypNA-pPNA/Pep-2. (b) In vitro RNase-H assay: 200 nM of cyclin B1 mRNA were incubated with a 50-fold excess of HypNA-PNAs (lane 2) or phosphorothioate oligonucleotide (lane 3) for 15 min at 37°C in the presence of E. coli Rnase H. Reaction products were analysed on 1.5% agarose gel. Control levels of untreated cyclin B1 mRNA are shown in lane 1.

mRNA. In contrast, no degradation of mRNA was observed with an excess of HypNA-*p*PNA (5 μM), demonstrating that, as already reported for PNAs and other chemically modified oligonucleotides, HypNA-*p*PNAs do not activate RNase-H. This is not surprising as only molecules whose structure is closest to that of DNA promote activation of RNase-H.[5,6] Instead, the mechanism through which PNAs contribute to down-regulation of gene expression is more likely to be directly associated with steric hindrance, as PNA binding to mRNA is known to disrupt ribosome assembly and block target protein synthesis.[1,4,6] Antigene activity of PNAs has been clearly demonstrated in cell-free systems. Strand invasion by PNAs has been demonstrated on supercoiled DNA using mixed sequences[37] and on relaxed dsDNA with polypyrimidine PNAs targeting polypurine motifs.[38–40] Moreover, although *in vivo*, there is little evidence for antigene activity of PNAs, several groups have reported that PNAs can induce down-regulation of target mRNA and have suggested either an antigene mechanism by interaction with target DNA, which inhibits the transcription machinery, or that PNA interaction with mRNA targets triggers its degradation through a hypothetical mechanism.[11–14] However, the PNAs used in these studies do not target homo-purine motifs, and would not be able to form a stable complex with DNA in order to block RNA polymerase.[10,38,39] Likewise, the antisense PNA that targets the first codons of the open reading frame of cyclin B1 gene should not be able to act as an antigene molecule. In the case of Pep-2, none of the mechanisms can be excluded, and we suggest that both steric hindrance and mRNA degradation are likely to come into play. However, nuclear Pep-2-mediated delivery of PNAs may also favour their interaction with the DNA target sequence.

Antisense cyclin B1 HypNA-pPNA inhibits proliferation of cancer cells

As delivery of antisense HypNA-*p*PNA directed against cyclin B1 promotes efficient downregulation of both cyclin B1 mRNA and protein levels, we asked to what extent this might affect proliferation of cancer cells. The effects of both the HypNA-*p*PNA chimera and of the corresponding classical PNA were investigated on the human breast cancer cell line MCF-7. Exponentially growing cells ($1–3 \times 10^4$) were incubated with different concentrations (0.5, 1, 2 μM) of HypNA-pPNA or PNA complexed or not with Pep-2 (molecular ratio 1:25) and cell proliferation was assessed 8 days postincubation. As shown in Figure 7, concentrations of 0.5 and 1 μM of HypNA-*p*PNA/Pep-2 consistently reduced cell proliferation by 70 and 92%, respectively. Similarly, classical PNA/Pep-2 complexes affected cell proliferation, although only by 35% for 1 μM and by 64% for 2 μM. In comparison, neither free HypNA-*p*PNA or PNA, scrambled molecules, or the peptide carrier alone affected cell proliferation, except for the highest concentrations of free HypNA-*p*PNA, which exhibited a slight decrease in cell proliferation. We cannot exclude that is in part associated with nonspecific cytotoxicity of PNA molecules, as already suggested by other groups.[10,21] Notwithstanding, these results emphasize the essential

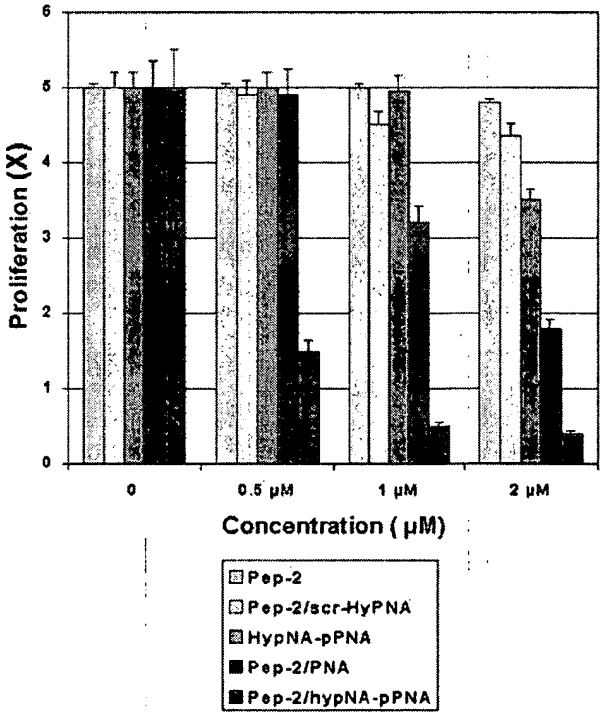

Figure 7 Inhibition of cancer cell proliferation by anti-cyclin B1 antisense HypNA-pPNA. MCF-7 cells were incubated with increasing concentrations of Pep-2, HypPNA-pPNA, HypPNA-pPNA/Pep-2, mismatched (ms) HypPNA-pPNA/Pep-2 and PNA/Pep-2 complexes on day 1 and cells were counted after 7 days of incubation. The values represent the average of four separate experiments.

role of Pep-2 in improving the bioavailability of antisense HypNA-*p*PNA and PNA molecules and demonstrate the selectivity and specificity of the antisense sequence used in this study.

Conclusions

PNAs have been proposed as an attractive alternative to classical antisense oligonucleotides.[5,6,10] However, in spite of their potent inhibitory features, their applicability in therapeutic treatments remains hampered by their low propensity to cross the cell membrane. In this study, we report a new strategy that combines the use of a chimeric antisense oligonucleotide mimic derived from a PNA (HypNa-*p*PNA) with a new noncovalent peptide-based delivery system, called Pep-2. We have shown that Pep-2 significantly improves the delivery of both PNAs and HypNA-*p*PNAs into several cell lines and reduces both the doses and the time required for them to induce a specific and robust biological response, thereby limiting nonspecific cytotoxic effects described upon treatment with high concentrations of PNAs.[11] Peptide-based carriers constitute very promising systems for the delivery of biologically active molecules into cells.[28,41] Pep-2 technology, in particular, presents several advantages including rapid delivery, lack of toxicity, insensitivity to serum and ability to release cargoes rapidly and therefore to enable rapid induction of a specific biological response. Moreover, as complex formation between Pep-2 and PNAs does not require any chemical coupling steps, the latter are not subject to any modifications, which might affect their biological properties, their specificity or their localization. We have specifically applied this technology to target an essential cell cycle protein, cyclin B1, and have proven its potential by demonstrating that we could specifically reduce both cyclin B1 protein and mRNA levels and arrest cell cycle progression in several cell lines with low concentrations of an antisense HypNA-*p*PNA designed to target cyclin B1. Downregulation of cyclin B1 gene expression mediated by Pep-2 delivery system is 100-fold more efficient than the effect mediated by classical cationic lipid methods. Although the peptide-based carrier Pep-2 is essential to improve the cellular uptake of the antisense molecule, it should be noted that the contribution of the HypNA-*p*PNA molecule is equally important, as approximately 10- and 25-fold greater concentrations of PNAs or of classical oligonucleotides were required to achieve a similar biological response. Finally, we have shown that Pep-2-mediated delivery of antisense cyclin B1 HyPNA-*p*PNA efficiently blocks cancer cell proliferation at very low concentrations, which suggests that this molecule is a good candidate for the development of a potential anticancer drug. Another advantage of Pep-2 may reside in its ability to the deliver HyPNA-*p*PNA and PNA into the nucleus, thereby favouring direct targeting of DNA.

In conclusion, these results demonstrate that the technology presented in this study constitutes an extremely powerful and specific means to target proteins and study their function in mammalian cells. Moreover, as Pep-2 enables the rapid delivery of HypNA-*p*PNAs into mammalian cells and consistently improves their potent antisense effect over a period of several days, due to the high stability of the chimera, it represents a very potent technology for administering antisense-based drugs in the context of therapeutic applications.

Materials and methods

Peptide synthesis and analysis

All peptides were synthesized by solid-phase peptide synthesis using AEDI-expansin resin with a 9050 Pep Synthesizer (Millipore, Wartford, UK) according to the Fmoc/tBoc method and purified as already described.[42] Pep-2 was synthesized with a cysteamide group at the C-terminus.

PNA and HypNA-pPNA synthesis

Phosphorothioate oligonucleotides and classical PNAs were obtained from Applied Biosystems (Foster City, CA, USA) and HypNA-*p*PNA were made by solid-phase synthesis using derivatized CPG support on a DNA synthesizer (Applied Biosystems 380B automated DNA synthesizer) as previously described.[25] To construct HypNA-*p*PNA oligomers, we synthesized dimer-building blocks as described earlier.[25,26] The solid-phase synthesis of an oligomer was carried out with phosphotriester coupling of dimer synthons. The phosphonate-protecting groups were removed by treatment with triethylammonium thiophenolate before final deprotection by ammonolysis. After desalting by gel filtration, an oligomer was purified by denaturing electrophoresis on 15% polyacrylamide gels plus 7 M urea with 100 mM Tris-borate/EDTA buffer (pH 8.3). The purity of the HypNA-*p*PNA oligomer was determined by analytical gel electrophoresis to be at least 85–90% and the concentration of oligomer was determined by ultraviolet absorbance at 260 nm.

Proteins and antibodies

Human cyclin B1, cyclin A and cdk2 protein were purified as already described.[28] Cyclin B1 mRNA were obtained as described in Peter *et al*.[33] Mouse monoclonal anti-cyclin B1 antibodies (SC-245) and rabbit polyclonal anti-Cdk2 antibodies (SC-163) for Western blotting were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell culture, Pep-2-mediated transfection

Adherent fibroblastic HS-68, 293, HeLa and MCF-7 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 1% antibiotics (streptomycin 10 000 µg/ml, penicillin, 10 000 IU/ml) and 10% (w/v) fetal calf serum (FCS), at 37°C in a humidified atmosphere containing 5% CO_2 as described previously.[28] For Pep-2-mediated delivery of PNA and HypNA-*p*PNA, HypNA-pPNA/Pep-2 and PNA/Pep-2 complexes were formed by incubation of different concentrations of antisense HypNA-*p*PNA or PNA from 0.05 to 5 µM with Pep-2 at a molecular ratio of 1:25, respectively, in 500 µl of DMEM for 30 min at 37°C. Cells grown to 60% confluence were then overlaid with these preformed complexes. After 30 min of incubation at 37°C, 1 ml of fresh DMEM supplemented with 10% FCS was added directly to the cells, without removing the overlay of PNA/Pep-2 or HypNA-*p*PNA/Pep-2 complexes, and cells were returned to the incubator.

Different concentrations of antisense HypNA-*p*PNA or phosphorothioate oligonucleotide were transfected with cationic lipids, lipofectamine or oligofectamine (Invitrogen, Carlsbad, CA, USA), according to the guidelines of the manufacturer. For cell cycle studies, HS-68 fibroblasts were synchronized by serum deprivation for 40 h, then restimulated to enter the cycle by addition of fresh DMEM supplemented with 20% FCS for 4 h. These cells were then incubated with HypNA-*p*PNA or HypNA-*p*PNA/Pep-2 complexes as described above. Cyclin B1 protein and mRNA levels were determined by Western and Northern blotting, respectively.

Flow cytometry analysis

HS68 or HeLa cells seeded onto 6-well plates were incubated with 1 μM Pep-2/HypNA-PNAs complex for 30 min at either 37°C or at low temperature (5°C). Cells were washed twice in PBS and incubated with trypsin (1 mg/ml) during 5 min at 37°C, washed, then finally resuspended in PBS. Experiments were performed on a FacsCalibur (Becton Dickinson) using the CellQuest software. For each individual sample, 10 000 cells were counted.

RNase-H assay

200 nM of cyclin B1 mRNA was incubated with a 50-fold excess of antisense HypNA-PNAs or phosphorothioate oligonucleotide in RNAse-H buffer (40 mM Tris-HCl pH 7.2, 4 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl and 1.25 U/μl Rnasin) for 15 min at 37°C in the presence of 0.5 U of *Escherichia coli* Rnase H (Promega). The reaction products were analysed on 1.5% agarose gel and stained with ethidium bromide as already described.[43]

Northern blotting

HS68 or HeLa cells (5×10^6 cells) were incubated with Pep-2/ HypNA-pPNA or HypNA-pPNA complexes. After 15 h, cells were collected and total RNA was isolated from cells with TriReagent™ (Sigma, Saint Louis, MO, USA) according to the manufacturer's instructions. RNA was then purified by phenol extraction followed by ethanol precipitation. RNA samples (10 μg) were separated by electrophoresis on formaldehyde agarose gels (1.2%), transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) and hybridized with ^{32}P-radiolabelled probes corresponding to the full-length cDNA of cyclin B1 or of GAPDH, respectively, prepared by random priming using the High Prime DNA labelling kit (Roche Diagnostics). Following hybridization and washing, the membrane was exposed to a Phosphorimaging screen and signals were acquired on a Phosphorimager (Molecular Dynamics).

Localization and immunofluorescence experiments

Cells were grown on acid-treated glass coverslips to 60% confluence. Cellular localization of FITC-labelled HyPNA-*p*PNAs was monitored by fluorescence microscopy in either living cells or cells fixed in paraformaldehyde (3%). For immunofluorescence experiments, cells were fixed in cold methanol 24, 48 or 72 h post-transfection, then washed in PBS, and neutralized in PBS/BSA (3% w/v). Cyclin B1 was detected using an anti-cyclin B1 antibody (cyclin B1, H-433, Santa Cruz Biotechnology Inc.) at a 1:100 dilution. Cell nuclei were stained with Hoechst 33 258 (1 μg/ml).

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